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14. ABSTRACT
In this final report we report that we successfully completed task # 1, after doing additional experiments requested by the reviewers. The manuscript is presently under editorial review in the American Journal of Pathology. In the past year we have concentrated mainly on Task # 2. The most significant reportable outcome of this task is that we have now removed doubts regarding the authenticity of fetuin-A as adhesion and growth signaling molecule. The purification protocol we have developed can purify fetuin-A to 100% purity. More importantly we have shown that human fetuin-A (ahsg) behaves exactly like its bovine counterpart. We have shown that in addition to mediating the activation of PI3 kinase/Akt, fetuin-A at high concentrations can also activate MAP kinases. We have also shown that in regular tissue culture, fetuin-A could be more relevant for cellular attachment than integrins. Fetuin-A is also actively internalized by the tumor cells but the significance of this internalization is presently unclear.

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Role of Fetuin-A in tumor Cell growth (Progress Report) W81XWH-07-1-0254

Josiah Ochieng, Ph.D. (Principal Investigator)

INTRODUCTION:

The central hypothesis of this grant was that **fetuin-A is a major serum derived growth factor for breast carcinoma cells and creates a favorable environment for the metastatic spread of tumor cells to the lungs.** We proposed two tasks to address the central Hypothesis: Task # 1. To elucidate the role of fetuin-A in mammary tumorigenesis as assessed by fetuin-A knockout and polyoma virus middle T antigen transgenic mice

(Months 1-18).
a) Start a breeding protocol by crossing Fet-/-C57/BL-6 mice with $Fet^{+/+}$PymT in FVB/N background (Months 1-5)
b) $Fet^{-/-}$PymT and $Fet^{+/+}$PymT mice will be moved from the breeding protocols to experimental groups **(Months 5-10)**.
c) Repeat task # 1b, to confirm data **(Months 10-15)**.
d) Analyze data (Months 15-29)
• Immunohistochemistry to show the expression of phosphorylated Akt, Phosphosmad2/3 in the tumors and other growth related signaling molecules **(Months 15-24).**
• To determine the influence of fetuin-A on the metastatic spread of breast tumors to the lungs **(Months 24-29).**

Task 1 has been completed and the manuscript is under editorial review in the American Journal of Pathology. Task # 2. The goal of Task 2 was to define the contribution of fetuin-A in the *in vitro* growth and signaling of human breast carcinoma cells (Months 18-36), and the sub-aims were to:

a) Purify fetuin-A from fetal bovine serum using a modified protocol. **(Months 18-24)**
b) Use the fetuin-A purified from fetal bovine to assay for growth and cell signaling in human breast carcinoma cells **(Months 24-30)**
c) Use the modified protocol to purify fetuin-A from human serum purchased from Sigma (Months 30-32) on breast tumor cell growth."

In this past reporting year, we have mainly concentrated on Task # 2. The following therefore is the summary of data obtained for Task # 2. We have completed most of the work and the manuscript dedicated to this task is almost ready for submission.

BODY:

Based on some of the suggestions of the reviewers who reviewed our initial submission, we evaluated a possible modulation of the p53 gene by fetuin-A. It has been reported that in the PymT transgenic mice, for tumors to develop, p53 has to be inactivated (1). Since lack of fetuin-A in these mice attenuates breast tumor development, there is a high possibility that fetuin-A inactivates either p53 or p19 (p14 in humans) to allow transformation and breast cancer progression. We therefore examined the expression of p53 and p19-ARF in the tumors of both wild-type and fetuin-A null animals and determined that indeed both p53 and p19 are down-regulated in the wild-type (presence of fetuin-A) but not in the null (absence of fetuin-A. The western blot of detergent solubilized proteins show that in the wild-type tissues (*Fet*$^{+/+}$) most of the p53 is targeted for degradation shown as poly-ubiquitinated p53 (Fig.1).

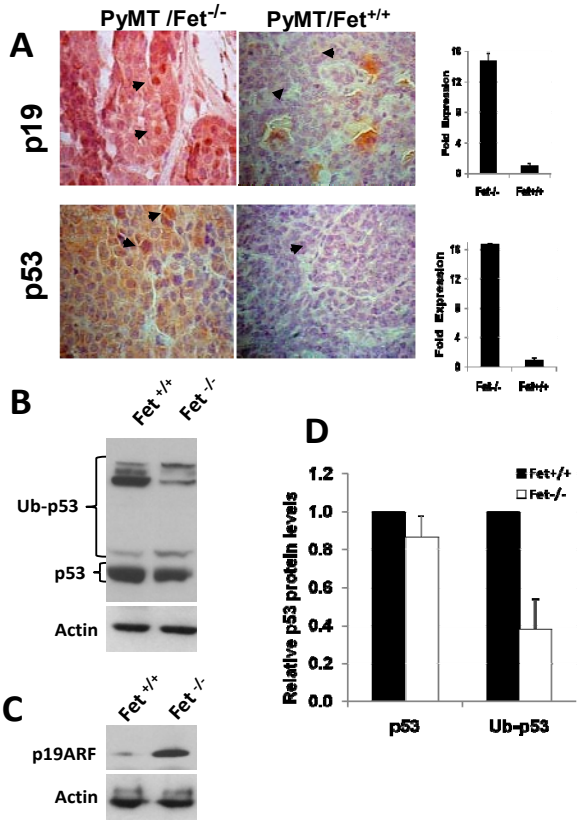

Fig 1. Fetuin-A mediated inactivation of ARF-p19 and degradation of p53.

The mammary tumor sections were probed with anti-p19 and anti-p53 antibodies (panel A). The fold-expression of P19 and P53 in *PyMT/Fet*$^{-/-}$ verses *PyMT/Fet*$^{+/+}$ tumor sections show a significant down-regulation of the tumor tumor suppressor proteins (panel A).

Equal amounts of detergent solubilized proteins of mammary tumors from the fetuin-A wild type and null mice prepared as described in "Materials and Methods" were separated on a 4-12% SDS gel, transferred to nitrocellulose membranes and the membranes probed with antibodies to p53 (panel B) or p19ARF (panel C). Actin was used as a loading control. Note that the exposure of membranes probed with anti-p53 antibody was carried out overnight at room temperature. Ub-p53 denotes ubiquitinylated p53. Panel D represents the densitometric analysis of the p53 and Ub-p53 bands of whole tissue lysates. Bars represent means and SD of densitometric ratios relative to *Fet*$^{+/+}$ from blots of two representative experiments.

To define the contribution of fetuin-A in the in vitro growth and signaling of human breast carcinoma cells (Months 18-36).

For a number of years, debate has raged as to whether fetuin-A is a major cellular attachment and growth promoting factor in culture medium, particularly media supplemented with fetal bovine serum (2). The main reason for this debate is the sticky nature of fetuin-A. Initially fetuin-A was purified by the Pedersen method. The Pedersen fetuin-A had cellular attachment properties but because it co-purifies with a number of proteins such as alpha-2-macroglobulin, a number of investigators argued that the cell attachment properties of fetuin-A could in fact be due to these impurities. This line of argument was further supported by the work of Spiro who purified fetuin-A to homogeneity and noticed that the purified protein lacked biological activity which is cell attachment in culture. This experiment sealed the fate of fetuin-A until the present studies. We are now happy to report that we have devised a purification technique for both human and bovine fetuin-A that does not sacrifice the biological activity of the protein. This technique was reported in the last progress report (2009). Using the glycerol gradient technique, we have been able to show that in human serum, the buoyant fractions that float on top of the gradient are the fractions that are enriched in fetuin-A and that they are the ones that support cellular attachment.

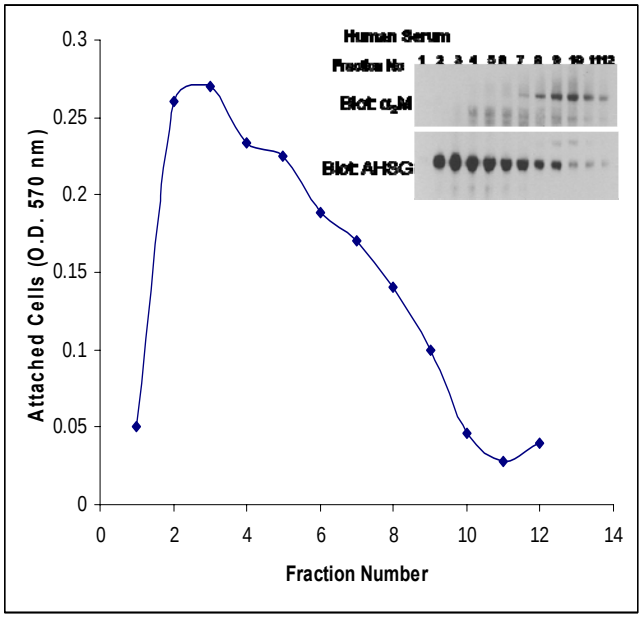

Fig 2. Attac hment of b reast tumor cells to h uman serum p roteins. Human exosome free serum was fractionated on a glycerol gradient and the wells of a micro-titer plate coated with aliquots of the gradient fractions (100 µl/well) overnight at 4°C. The excess proteins were removed and the wells washed once with SFM and BT-549 cells added to the wells at 2 x10^4 cells/well in SFM containing 1 mM Ca^{2+}. The cells were allowed to attach for 3h at 37°C and the non-adherent cells washed off. The cells were incubated again in SFM containing Alamar Blue. After 6-8 h, the plates were read at 570 nm to determine the number of viable cells. Insert depicts the western blot of the fractions probed with anti-AHSG and α2M.

We next questioned whether the attachment of breast tumor cells in the presence of culture medium supplemented with 10% fetal bovine serum is as sensitive to calcium chelation as the attachment of the same cells to wells coated with fetuin-A. The goal of this experiment was to prove that Ca^{2+} is more relevant for attachment of breast tumor cells in tissue culture compared to either Mg^{2+} or Mn2+ ions. Tumor cells BT-549 were therefore allowed to attach to micro-titer wells (2 x 10^4 cells/well) in the presence of either complete medium or fetuin-A in

serum free medium (SFM) at 0.25% w/v. This is the same concentration of fetuin-A as in complete medium. Complete medium containing 10% serum has all the three ions while SFM containing 1 mM Ca^{2+} lacks both magnesium and manganese. As a control, we also coated some wells with fibronectin to which tumor cells adhere in the presence of magnesium or manganese. The same number of cells was added to these wells coated with fibronectin. After the attachment, the cells were incubated in the presence of Hanks buffered salt solution (HBSS) containing EGTA (0-1 mM) for 30 min. The detached cells were washed twice with HBSS containing 1 mM Ca^{2+} and the adhered cells incubated in the respective medium containing Alamar Blue for 4-6 h or until color change to determine the number of attached cells. When we did this experiment, it was clear to us that in tissue culture medium, the cells use mainly Ca^{2+} for attachment to serum proteins for which Ca^{2+} is necessary, We have shown that attachment of cells to fetuin-A requires Ca^{2+}. The attachment of cells to fibronectin requires manganese or magnesium which is less sensitive to EGTA chelation and therefore more cells are attached in the presence of EGTA.

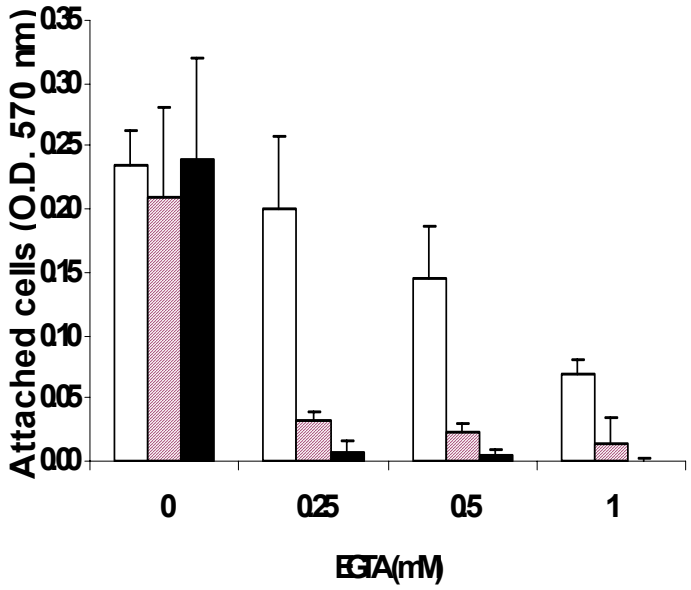

Fig 3. The significance of Ca^{2+} to the attachment of tumor cells in vitro. The BT-549 cells were allowed to attach to the wells of a micro-titer plate in either complete medium (red bars) or 0.25% fetuin-A in SFM (solid black bars) for 6 h. Some of the wells were coated with fibronectin (2 µg/well) and the same number of cells added in complete medium (open bars) for 6 h. The wells were then washed once with HBSS containing divalent ions and then incubated with HBSS containing EGTA (0-1 mM) for 30 min. The wells were washed once more with HBSS and finally the adhered cells were incubated in the respective medium containing Alamar Blue for 4-6 h or until color change to determine the number of cells.

Growth signaling mediated by Fet/Ca^{2+} in tumor cells.

In our earlier work, we reported that fetuin-A mediates the PI3 kinase/Akt signaling in tumor cells. However, since we had used Pedersen fetuin-A in these studies, it was not clear whether the signaling was due to fetuin-A per se and not

alpha-2-macroglobulin, the major contaminant of fetuin-A. Moreover, other studies have shown that alpha-2-macroglobulin can also activate the PI3 kinase/Akt pathway. Therefore in Task 2 b, our goal was to purify fetuin-A to homogeneity and determined whether it can mediate the activation of the pathway in breast tumor cells.

For this study we used three purified fractions of Pedersen fetuin-A (purified by glycerol gradient purification strategy). Fraction S1 was the purest homogenous fraction of fetuin-A that is detected as one band both by colloidal coomassie and western blot analysis. Fraction S2 was slightly contaminated by inter-alpha globulins (Fig. 3) while fraction 3 was mainly alpha-2-macroglobulin.

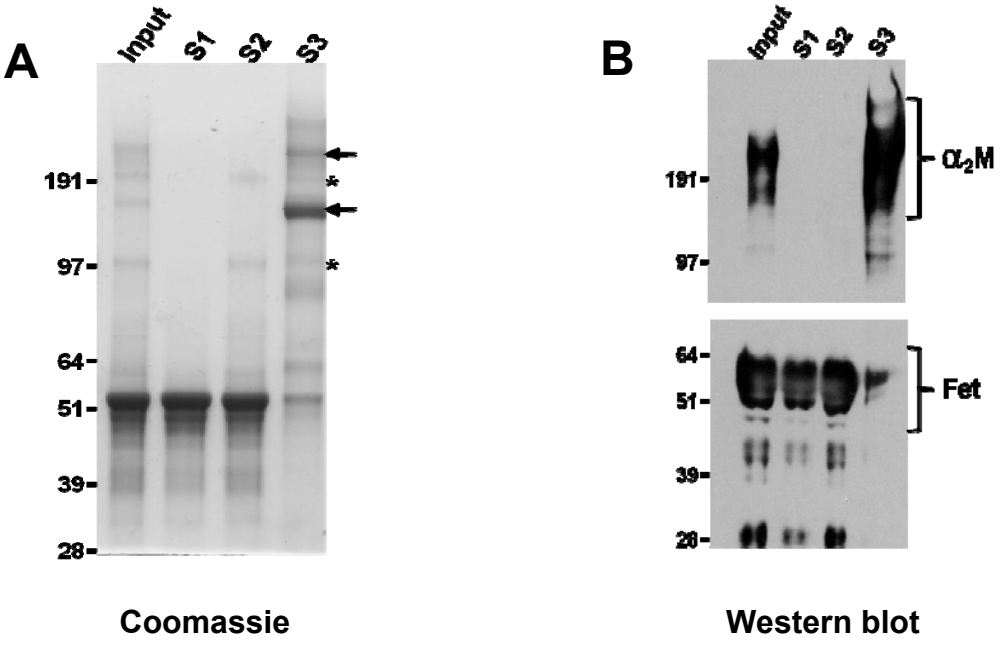

Fig. 4. Analysis of glycerol gradient purified fetuin-A. In panel A, glycerol fractionated fetuin-A fractions were pooled and named S1 (purest), S2 (contaminates slightly with inter alpha inhibitor H2 globulin (asterisks), and S3 (mainly alpha-2-macroglobulin). The pooled fractions were exhaustively dialyzed against HBBS at 4 °C. Equal amounts of the pooled fractions were analyzed by SDS-PAGE in 4-12% gradient gels and either stained with colloidal Coomassie (A) or blotted unto nitrocellulose membranes and probed with antibodies to fetuin-A or α_2M (B).

The fetuin-A fractions (S1, S2, S3) were incubated with BT-549 cells for 10 min in the absence or presence of graded doses of the proteins and then monitored the MAP kinase and PI3 kinase/Akt pathways by measuring the levels of phospho-ERK1/2 and P-Akt (473) as readouts. Erk1/2 was activated in the presence of 2 mM Ca^{2+} and this activation was depressed in the presence of low

concentrations of S1 (0.05-0.1 mM). However at higher concentrations of S1(>0.3 mg/ml), Erk was activated in the presence of Ca^{2+} (Fig. 7A and B) . Higher concentrations of S1, S2 and S3 (0.3 mg/ml) were able to activate ERK to the same extent in the presence of Ca^{2+}. PI3 kinase/Akt on the other hand was activated by fetuin-A fractions in a dose dependent manner (Fig. 7A and B). The fetuin-A fractions S1,S2, and S3 were all capable of activating this pathway to the same extent (Fig 7A). As a control, we also used in these assays, the BT-A6A cell line in which annexin-6 has been knocked down. Annexin-6 is one the cell surface proteins that interact with fetuin-A (3). Interestingly, BT-A6A, showed almost negligible activity of the PI3 kinase/Akt pathway (Fig. 7A). However, MAP kinase pathway was highly active in this cell line, even in the absence of Ca^{2+} (Fig. 7B).

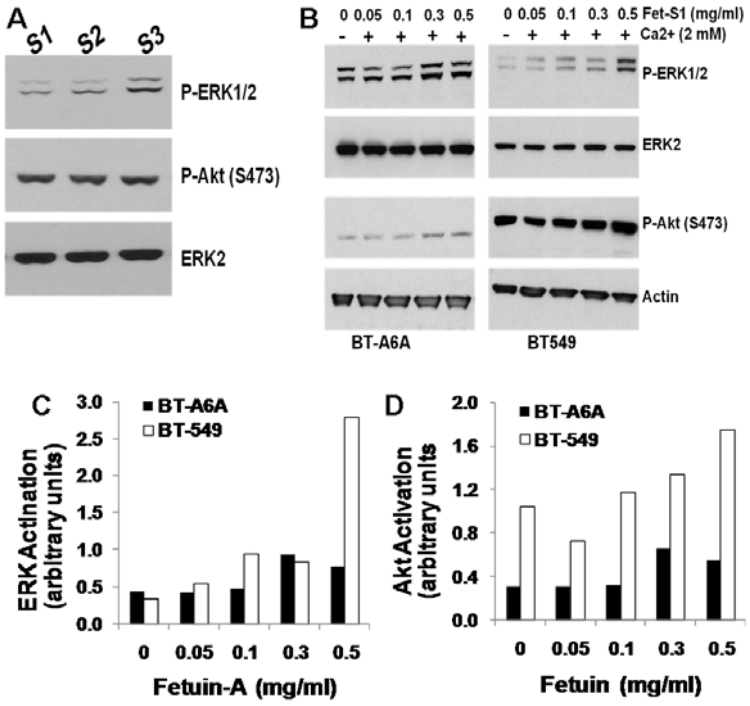

Fig. 5. F**etuin-A -dependent a ctivation of M APK in brea st carcinoma cells requi res annexins and extracellular Ca^{2+}**. A) BT549 cells were serum-starved for 24 h, then washed 2x with HBS and incubated in HBS with or without 2 mM Ca^{2+} and the indicated concentrations of the pooled glycerol gradient fractions of fetuin-A. Cells were harvested using a cell scrapper, washed in ice-cold HBS and lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of proteins were analyzed by western blotting using antibodies to phospho-ERK1/2, phospho-Akt (S473) and total ERK2 as the loading control. B) Serum-starved BT549 cells or annexin A6-depleted BT549 cells (BT-A6A) were washed with HBBS and incubated in HBBS with or without 2 mM Ca^{2+} and the indicated concentrations of purified fetuin-A (fraction S1). Cells were harvested using a cell scrapper, washed in ice-cold HBBS and lysed

in ice-cold RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of proteins were analyzed by western blotting using antibodies to phospho-ERK1/2 and phospho-Akt (S473). Total ERK2 and β-actin were used as the loading control. C-D) Densitometric analysis of ERK and Akt phosphorylation in the total cell lysates. Bars represent ERK1/2 phosphorylation normalized to total ERK2 (panel C) or Akt phosphorylation normalized to β-actin (panel D) from a representative experiment .

Uptake of fetuin-A by breast epithelial cells. Having reported the uptake of fetuin-A by breast tumor cells in the second progress report (2009), we have continued to explore the significance of this uptake. We report here that fetuin-A is up-taken mainly by cells in their log phase of growth and not when they are confluent. This therefore means that the uptake is cell cycle dependent with the implications that the uptake is critical for growth. Because of the significance of this observation, this is the direction we see this project taking from now onwards. We are currently looking at the intracellular binding partners for fetuin-A by Tap tagging to determine what potential role it may play in the growth of breast tumor cells.

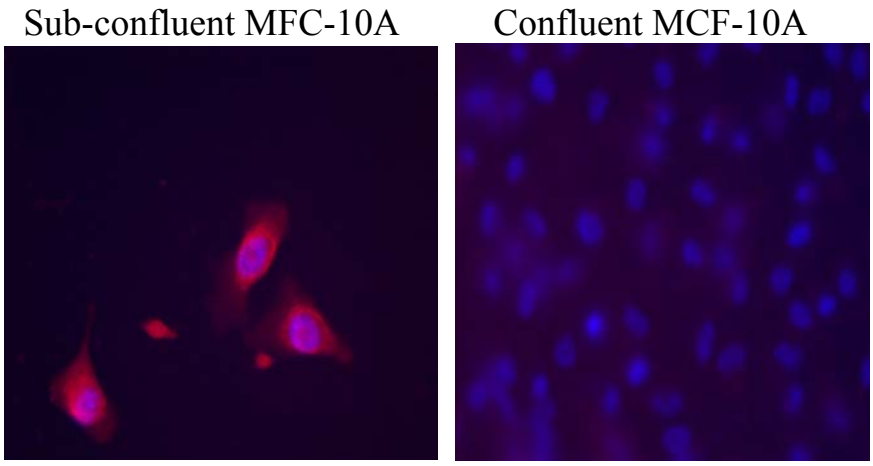

Fig 6. Uptake of labeled fetuin-A by a breast epithelial cell line, MCF-10A. MCF-10A was allowed to grow until approximately 30% confluent (sub-confluent) or until 100% confluent in complete medium on glass cover-slips. The medium was then replaced with SFM containing Ca^{2+} ions at 37^{o}C. Once the cells stabilized at this temperature, rhodamine isothiocynate labeled fetuin-A was added to both the sub-confluent and confluent cells and allowed to incubate for 10 min. The cells were then washed twice in cold SFM, fixed and then examined and photographed (epifluorescence microscopy). The nuclei stain blue with DAPI.

KEY RESEARCH ACCOMPLISHMENTS:

- We have demonstrated that the uptake of fetuin-A by tumor cells could be just as important as its role as an extracellular adhesion molecule.

- We have demonstrated that in Fetuin-A in can modulate the p53/ARF signaling pathway that is necessary for in vivo tumorigenesis.

- We conclusively demonstrate that human fetuin-A, just like its bovine counterpart, is also able to mediate the attachment and growth signals in breast epithelial cells.

Reportable Outcomes:

- A major manuscript describing the work completed in specific aim # 1 is under editorial review in the American Journal of Pathology.

- A major manuscript describing work done in Task # 2 is will be submitted for publication next month (April 2010).

- This award has supported the Ph.D. dissertation of one minority student Bobby Guillory who will defend his thesis by May of this year (His graduation has been delayed pending the acceptance of the paper that is under editorial review)

Conclusion:

In conclusion, the research experience resulting from this grant has been highly rewarding. We are now in the process of preparing R01 grant application to NIH based on the data of this award. It has become clear to us that indeed fetuin-A is an important adhesion and growth signaling molecule in serum. Reports that have shown that the concentration of fetuin-A in serum is depleted in cases where there is a high tumor load could mean that fetuin-A is rapidly up-taken by tumor cells for growth. This is one aspect that we would like to uncover in our next grant application. Once again as the PI of this grant, I am extremely thankful to the DOD for this opportunity. New data useful in the fight against cancer will be realized by this research effort.

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